(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
6 December 2001 (06.12.2001)

PCT

(10) International Publication Number
WO 01/92561 A2

(51) International Patent Classification[7]: C12Q 1/00

(21) International Application Number: PCT/US01/18062

(22) International Filing Date: 1 June 2001 (01.06.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/209,058 2 June 2000 (02.06.2000) US

(71) Applicant *(for all designated States except US)*: **PURDUE RESEARCH FOUNDATION** [US/US]; 1291 Cumberland Avenue, West Lafayette, IN 47906 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **PITTENDRIGH, Barry, Robert** [US/US]; Apartment #1316, 400 North River Road, West Lafayette, IN 47906 (US). **MURDOCK, Larry, Lee** [US/US]; 3527 Creek Ridge, Lafayette, IN 47905 (US). **GAFFNEY, Patrick, Joseph** [GB/GB]; 1203 Chandler Street, Madison, WI 53715 (GB).

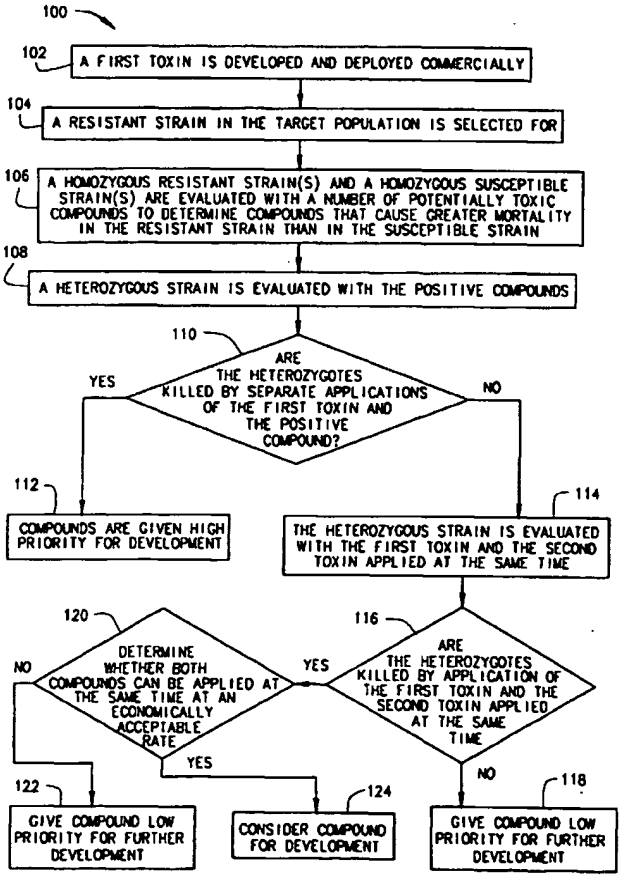
(74) Agents: **BEULICK, John, S.** et al.; Armstrong Teasdale LLP, Suite 2600, One Metropolitan Square, St. Louis, MO 63102-2740 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European

[Continued on next page]

(54) Title: METHOD OF SCREENING FOR NEGATIVE CROSS RESISTANCE



(57) Abstract: A method of evaluating the efficacy of molecules against a target population including a strain resistant to a first toxin includes determining a susceptible strain in the target population and selecting for the resistant strain in the target population. The susceptible strain being susceptible to the first toxin and the resistant strain being resistant to the first toxin. The method further includes evaluating the efficacy of the resistant strain with a plurality of molecules to determine a second toxin that is more toxic to the resistant strain than to the susceptible strain, evaluating the efficacy of a heterozygous strain of the target population with separate applications of the first toxin and the second toxin, and assigning a priority rating to the second toxin if the separate applications of the first toxin and the second toxin are at least as toxic to the heterozygous strain as to the susceptible strain.

WO 01/92561 A2

patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

METHOD OF SCREENING FOR NEGATIVE CROSS RESISTANCE

BACKGROUND OF THE INVENTION

This invention relates generally to negative cross resistance, and more particularly, to evaluating the efficacy of molecules to determine molecules that exhibit negative cross resistance.

Two of the most important scientific events of the twentieth century have been the green revolution and the development of antibiotics. The green revolution, with the large-scale use of insecticides and herbicides, has brought about dramatic increases in quantity and quality of food for an ever-growing human population, allowing for reliable food sources for billions of people on this planet. In turn, antibiotics have dramatically reduced the mortality rates of the human population to bacterial diseases, virtually wiping out bacterial epidemics. However, resistance has evolved due to the large scale use of the insecticides, herbicides, and antibiotics.

Although efforts have been made to slow the development of resistance to pesticides and antibiotics, the evolution of resistance is generally considered inevitable. Once widespread resistance develops, the chemical (or chemical-class) that resistance has developed against is typically abandoned. The subsequent focus in the research and industrial community is to identify novel pesticides and antibiotics with different modes of action, where positive cross-resistance to previously used biocides does not occur. One alternative to discarding old compounds and continually seeking new compounds is the development of negative cross-resistance strategies to control organisms containing the resistance allele.

Negative cross-resistance (NCR) as a strategy for insecticide resistance management refers to a scenario where organisms tolerant to one compound are highly sensitive to another compound and vice versa. For example, if one treats an insect population with a toxin such as pesticide 'A', the number of insects carrying alleles resistant to pesticide 'A' will increase in frequency. After numerous generations, insects carrying the resistance allele will comprise the majority of the

BRIEF SUMMARY OF THE INVENTION

In one aspect of the invention, a method of evaluating the efficacy of a molecule against a target population, the target population strain including a strain resistant to a first toxin, comprises determining a susceptible strain in the target population and selecting for the resistant strain in the target population. The susceptible strain being susceptible to the first toxin and the resistant strain being resistant to the first toxin. The method further comprises evaluating the efficacy of the resistant strain with a plurality of compounds to determine a second toxin that is more toxic to the resistant strain than to the susceptible strain, evaluating the efficacy of a heterozygous strain of the target population with separate applications of the first toxin and the second toxin, and assigning a priority rating to the second toxin if the separate applications of the first toxin and the second toxin are at least as toxic to the heterozygous strain as to the susceptible strain.

In another aspect, a method is provided for testing for negative cross resistance in a target population. The method comprises determining a susceptible strain (S/S) in the target population, the susceptible strain (S/S) susceptible to a first toxin, selecting for a resistant strain (R/R) in the target population, the resistant strain (R/R) resistant to the first toxin, evaluating the efficacy of the resistant strain (R/R) with between about 10 and 10^9 molecules to determine a second toxin that is more toxic to the resistant strain (R/R) than to the susceptible strain (S/S), evaluating the efficacy of a heterozygous strain (R/S) of the target population with separate applications of the first toxin and the second toxin to determine if separate applications of the first toxin and the second toxin are at least as toxic to the heterozygous strain (R/S) as to the susceptible strain (S/S), and assigning a high negative cross resistance priority to the second toxin if the separate applications of the first toxin and the second toxin are at least as toxic to the heterozygous strain (R/S) as to the susceptible strain (S/S).

In another aspect, a method is provided for screening chemicals to determine a commercialization prioritization for the compounds. The method comprises determining a susceptible strain (S/S) in a target population, the susceptible

strain (S/S) susceptible to a first toxin, selecting for a resistant strain (R/R) in the target population, the resistant strain (R/R) resistant to the first toxin, testing the resistant strain (R/R) with a number of molecules to determine at least one chemical that is more toxic to the resistant strain (R/R) than to the susceptible strain (S/S), testing a heterozygous strain (R/S) of the target population with separate applications of the at least one chemical and the first toxin to determine if the at least one chemical and the first toxin are at least as toxic to the heterozygous strain (R/S) as to the susceptible strain (S/S), assigning a high priority to the at least one molecule if the at least one molecule and the first toxin are at least as toxic to the heterozygous strain (R/S) as to the susceptible strain (S/S), testing the heterozygous strain (R/S) with both of the at least one molecule and the first toxin applied at the same time to determine if the combination of the at least one molecule and the first toxin is at least as toxic to the heterozygous strain (R/S) as to the susceptible strain, determining whether both the at least one molecule and the first toxin can be applied to the target population at the same time at an acceptable rate, and assigning a high priority to the at least one molecule if the at least one molecule and the first toxin can be applied to the target population at the same time at an economical rate.

In another aspect, a method is provided for managing resistant alleles in a target population. The method comprises determining a susceptible strain in the target population, the susceptible strain being susceptible to the first toxin, selecting for a resistant strain in the target population, the resistant strain including at least one resistant allele, the resistant allele providing resistance to the resistant strain against the first toxin, screening the resistant strain with a plurality of molecules to determine a second toxin that is more toxic to the resistant strain than to the susceptible strain, screening a heterozygous strain of the target population with separate applications of the first toxin and the second toxin, and prioritizing the second toxin for commercialization testing if the separate applications of the first toxin and the second toxin are at least as toxic to the heterozygous strain as to the susceptible strain.

In another aspect, a method is provided for managing a tract of land against a resistant strain of a target population. The method comprises determining a susceptible strain in a target population, the susceptible strain susceptible to the first

toxin, selecting for the resistant strain which is resistant to the first toxin, evaluating the resistant strain to determine a second toxin that is more toxic to the resistant strain than to the susceptible strain, evaluating a heterozygous strain of the target population with separate applications of the first toxin and the second toxin, and utilizing the second toxin to manage the land against the resistant strain if the separate applications of the first toxin and the second toxin are at least as toxic to the heterozygous strain as to the susceptible strain.

In another aspect, a method is provided for managing a portfolio that includes a plurality of molecules. The method comprises selecting a target population, obtaining a first strain in the target population, the first strain susceptible to a first toxin, obtaining a second strain in the target population, the second strain resistant to the first toxin, evaluating the efficacy of the second strain with multiple molecules in the portfolio to determine multiple second toxins that are more toxic to the second strain than to the first strain, evaluating the efficacy of a third strain of the target population with separate applications of the first toxin and the second toxins, the third strain heterozygous for resistance to the first toxin, and prioritizing the second toxins based on their performance in the second strain evaluation and in the third strain evaluation.

In another aspect, a method is provided for assessing whether to develop a molecule included within a molecule portfolio. The method comprises evaluating the efficacy of a resistant strain of a target population with molecules in the molecule portfolio, selecting a molecule that is more toxic to the resistant strain than to a susceptible strain, the susceptible strain susceptible to a first toxin, the resistant strain resistant to the first toxin, evaluating the efficacy of a heterozygous strain of the target population with separate applications of the first toxin and the selected molecule, the heterozygous strain heterozygous for resistance to the first toxin, and selecting the molecule for commercialization if the separate applications of the first toxin and the selected molecule are at least as toxic to the heterozygous strain as to the susceptible strain.

In another aspect, a method is provided for controlling an insect population on a tract of land. The method comprises obtaining an insect strain susceptible to a first toxin, obtaining an insect strain resistant to the first toxin, screening the resistant insect strain with a plurality of molecules, determining a second toxin that is more toxic to the resistant insect strain than to the susceptible insect strain, screening a heterozygous insect strain with separate applications of the first toxin and the second toxin, determining if separate applications of the first toxin and the second toxin are at least as toxic to the heterozygous strain as to the susceptible strain, and utilizing the second toxin to control the insect population on the tract of land if separate applications of the first toxin and the second toxin are at least as toxic to the heterozygous insect strain as to the susceptible insect strain.

In another aspect, a method is provided for utilizing a pest management system to manage a pest population. The system including a plurality of molecules. The method comprises determining a susceptible strain in the pest population, the susceptible strain susceptible to a first toxin, selecting for a resistant strain in the pest population, the resistant strain resistant to the first toxin, evaluating the resistant strain to determine a second toxin that is more toxic to the resistant strain than to the susceptible strain, evaluating a heterozygous strain with the first toxin and the second toxin applied at the same time, and utilizing the second toxin to manage the pest population if the application of the first toxin and the second toxin at the same time are at least as toxic to the heterozygous strain as to the susceptible strain.

In another aspect, a method is provided for utilizing a pest management system to manage a pest population. The system including a plurality of molecules. The method comprises determining a susceptible strain in the pest population, the susceptible strain susceptible to a first toxin, selecting for a resistant strain in the pest population, the resistant strain resistant to the first toxin, evaluating the resistant strain to determine a second toxin that is more toxic to the resistant strain than to the susceptible strain, evaluating a heterozygous strain of the target population with separate applications of the first toxin and the second toxin, and utilizing the second toxin to manage the pest population if the separate applications of the first toxin and

the second toxin are at least as toxic to the heterozygous strain as to the susceptible strain.

In another aspect, a method is provided for utilizing a pest management system that includes a plurality of molecules. The method comprises selecting a pest population, obtaining a first strain in the pest population, the first strain susceptible to a first toxin, obtaining a second strain in the pest population, the second strain resistant to the first toxin, evaluating the efficacy of the second strain with multiple molecules in the system to determine multiple second toxins that are more toxic to the second strain than to the first strain, evaluating the efficacy of a third strain of the pest population with separate applications of the first toxin and at least one second toxins, the third strain heterozygous for resistance to the first toxin, and selecting at least one of the at least one second toxin to manage the pest population based on the performance of the selected at least one of the at least one second toxin in the second strain evaluation and in the third strain evaluation.

In another aspect, a method is provided for utilizing a pest management system that includes a plurality of molecules. The method comprises selecting a pest population, obtaining a first strain in the pest population, the first strain susceptible to a first toxin, obtaining a second strain in the pest population, the second strain resistant to the first toxin, evaluating the efficacy of the second strain with multiple molecules in the system to determine multiple second toxins that are more toxic to the second strain than to the first strain, evaluating the efficacy of the third strain with the first toxin and at least one second toxin applied at the same time, and selecting at least one of the at least one second toxin to manage the pest population based on the performance of the selected at least one of the at least one second toxin if the application of the first toxin and the second toxin at the same time are at least as toxic to the third strain as to the first strain.

In another aspect, a method is provided for evaluating a molecule for negative cross resistance. The method comprising using a *Rst(2)DDT* locus in Drosophila melanogaster as a resistance locus for use in negative cross resistance screens for molecules capable of controlling metabolic resistance to insecticides.

In another aspect , a method is provided for evaluating molecules for negative cross resistance. The method comprising using a putative target site locus in Drosophila melanogaster as a resistance locus for use in negative cross resistance screens for molecules capable of controlling metabolic resistance to insecticides.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graphic illustration of the concurrent use of two toxins that are negative cross resistance factors on a target population where both toxins cause equal mortality in homozygous wild-type and mutant insects, and the heterozygotes are unaffected by the combination.

Figure 2 is a graphic illustration of the concurrent use of two toxins that are negative cross resistance factors on a target population where the two toxins cause equal homozygous mortality rates and the heterozygotes have higher mortality rates than the homozygotes.

Figure 3 is a graphic illustration of the concurrent use of two toxins that are negative cross resistance factors on a target population where the first toxin causes a 99% mortality rate to the homozygous wild-type insects, the second toxin causes a 70% mortality rate to the homozygous resistant insects, and 70% of the heterozygous target population are killed.

Figure 4 is a graphic illustration of the concurrent use of two toxins that are negative cross resistance factors on a target population where both toxins cause a 70% mortality rate in the homozygous wild-type and mutant insect population, and the heterozygotes are either unaffected by the combination or they are much more susceptible.

Figure 5 illustrates the effect of starting allelic frequency on the fate of the resistance allele in the presence of two negative cross resistance factors that kill both homozygous lines (susceptible and resistant) at a 50% level and the heterozygotes at a 70% level.

Figure 6 illustrates a method of screening for compounds to be deployed commercially as NCR factors.

Figure 7 is a Northern blot of Canton-S (Can-S), DDT-resistant line with no exposure to DDT (Rst(2)DDT-Wisconsin), and the DDT-resistant fly line with exposure to 20 μg /vial of DDT for 24 hours (Rst(2)DDT-Wisconsin–20 μg of DDT).

Figure 8 illustrates a method of providing management decisions for the development of NCR factors.

Figure 9 is a graphical illustration of the application of deltamethrin, DDT, and the combination of deltamethrin and DDT at the same time to *para*^{tsl} flies, Canton-S flies, and heterozygotes.

DETAILED DESCRIPTION OF THE INVENTION

Exemplary embodiments of systems and methods that facilitate evaluating the efficacy of molecules to determine negative cross resistance factors are described below. The systems and methods facilitate, for example, evaluating the efficacy of molecules against a target population including a strain resistant to a first toxin, evaluating the efficacy of molecules for negative cross resistance in a target population, and evaluating the efficacy of molecules to determine an advancement order for advancement to additional evaluation regarding commercialization prioritization for the compounds. Although the evaluation methods are often described in terms of an entire process, it should be understood that each evaluation method can be used alone, or in combination with any of the other evaluations described hereinafter.

As used hereinafter, the term molecules includes, but is not limited to, natural molecules, synthetic molecules, chemicals, compounds, biotechnical species, and biotechnical moieties. In addition, evaluating the efficacy includes testing, screening, and determining. Further, target population includes a pest population which includes any living organism growing where it is unwanted, including, but not limited to, a weed population, a bacterial population, an insect population, a fungus

population, a virus population, and a population of disease contributing organisms living in a body of a mammal.

A population strain, and in particular, an insect strain, is defined as at least one of a genotype, a phenotype, a genotype and a phenotype, a group of genotypes, a group of phenotypes, and a group of genotypes and phenotypes, that display a response to a toxin in terms of a life history parameter. A life history parameter is defined in terms of mortality rates (Lethal time 50 (LT50) or lethal dose 50 (LD50)) or developmental time or other terms. In one embodiment, a strain contains at least one genotype or phenotype that is resistant or susceptible. Resistance and susceptibility are relative terms and are defined in relation to each other (resistance ratio). Resistance is defined as a genotype or phenotype that requires high rates of application to achieve high mortality rates or in which high rates of application of the chemicals do not result in high mortality rates in the resistant strain. High rates, in practical terms include levels of application of the toxin above the acceptable label rate as defined by regulatory agencies.

In one embodiment, a resistance ratio is the LD50 of resistant insects (strain 1) divided by the LD50 of susceptible insects (strain 2). For example:

Resistance Ratio = LD50 of strain 1 divided by LD50 of strain 2

The resistance ratio can be provided as a number (e.g. 2) or as a ratio 2:1. In one embodiment, a resistance ratio indicating resistance is defined, between strain 1 and strain 2 as 1.5:1 or greater (e.g. 10,000,000:1 ratio). This ratio covers a broad range of toxic ratios. For example, a 1:10,000,000 ratio indicates that the resistant insect could walk on crystals of the toxin without being impacted.

Table 1 illustrates the concurrent use of two toxins to minimize both the frequency of resistance alleles as well as the size of a target population, for example, an insect population. Table 1 shows the impact of the toxicity of the second toxin on both the change in allelic frequency of the resistance allele and the effective control of the insect population. In the example illustrated in Table 1, the first toxin causes a 99% mortality rate in the homozygous wild-type insects.

Table 1

Mortality Rate of		50% Allelic Frequency	
Homozygous Resistant	Heterozygous	Days to	Mean Population Size
1	1	100.5	9368
	10	100.5	7446
	50	109.0	2342
	90	138.0	328
	99	260.5	99169
50	50	117	2569
	70	123	1178
	90	152	171
	99	275	3287
90	90	191.5	81
	99	333	2
99	99	Population Crashed	

If both toxins do not have similar levels of toxicity on the homozygous lines they impact, the allele susceptible to the least toxic compound will increase in frequency. As the allele susceptible to the least toxic compound becomes more common, the toxin-pair becomes less effective in controlling the insect population. In addition, both toxins must kill the heterozygous and homozygous insects at a high rate in order to keep the insect population below economic thresholds.

Table 2 illustrates the concurrent use of two toxins where the homozygous mortality rates are equal for the two compounds and the heterozygotes have either lower or higher mortality rates than the homozygotes.

Table 2

Mortality Rate of Homozygous Resistant/Susceptible Genotypes	Heterozygote Mortality	Fate of Resistance Allele at the End of Test
99% / 99%	1%	Asymptotic to 50%

	90%	Asymptotic to 50%
	99%	Population Crashed
50% / 50%	5%	27.3%
	10%	20.9%
	50%	0.14%†
	70%	Asymptotic to 0.025%†
	99%	Asymptotic to 0.011%†
10% / 10%	10%	0.14%†
	50%	Asymptotic to 0.023%†
	90%	Asymptotic to 0.278%†

† High probability of resistance allele going to extinction.

Figures 1 through 4 graphically illustrate the concurrent use of two toxins that are negative cross resistance factors on a target population, such as an insect population. If the combined toxins kill more heterozygotes than homozygotes, the allelic frequency of mutant alleles tend to go to an extreme value, i.e., either close to fixation or close to extinction depending on initial frequency. In Figure 1, both toxins cause equal mortality in homozygous wild-type and mutant insects, but the heterozygotes are unaffected by the combination (1% heterozygous mortality). In Figure 2, the two toxins cause equal mortality rates in the homozygous lines and the heterozygotes have higher mortality rates than the homozygotes.

When the combined toxins kill fewer heterozygotes than homozygotes, the allelic frequency of the two alleles approaches a 50% equilibrium point. In Figure 3, the first toxin causes a 99% mortality rate to the homozygous wild-type insects, and the second toxin causes a 70% mortality rate to the homozygous resistant insects. In addition, 70% of the heterozygous target population are killed (in addition to the background mortality rate). As illustrated in Figure 3, when the frequency of the resistance allele becomes greater than 50%, the population begins to expand. In Figure 4, both toxins cause a 70% mortality rate in the homozygous wild-type and mutant insect population, but the heterozygotes are either unaffected by the

combination (1% heterozygote mortality) or they are much more susceptible (99% heterozygote mortality).

If the allelic frequency of the two alleles approaches a 50% equilibrium, the pesticide combination is not effective in controlling the insect population. It is important to note that high heterozygous fitness as compared to both homozygotes is rare in nature. Since heterozygous fitness in the presence of both toxins has not typically been investigated in field and laboratory examples of NCR it is difficult to assess how rare the phenomenon is. In cases where high heterozygote fitness is observed in screens to discover NCR factors, such toxins should typically be given low priority for commercial development.

High heterozygote mortality rates result in the resistance allele becoming very rare in the population or extremely common in the population, depending on the starting allelic frequency. Since the frequency of the resistant allele is an issue of probability in a field situation, in which there are many variables, it is quite possible that with a starting allelic frequency (for the resistance allele) below 50%, the resistance allele may occasionally tend to high frequency when the two toxins are used. Thus, in a field scenario, it is beneficial to initially use a single toxin to drive one allele to a more extreme value, to increase the probability of a preferred allele being the most common.

For example, assume that there is a practical reason that requires keeping the resistance allele at a low frequency. If the starting allelic frequency of resistance is greater than 10% one could use the second NCR factor (which kills the resistant insects preferentially) to reduce the frequency of the resistance allele before using the two toxins concurrently. Thus, by first applying the toxin that preferentially kills the resistant insect, one biases the probability of keeping the resistance allele at a low frequency when the two toxins are used together (See, for example, Figure 5). Once resistance is driven to a low frequency, the single toxin can again be used. Specifically, Figure 5 illustrates the effect of starting allelic frequency on the fate of the resistance allele in the presence of two negative cross resistance factors that kill both homozygous lines (susceptible and resistant) at a 50% level and the

heterozygotes at a 70% level. The X-axis represents the time (in days) from the start of the experiment and the Y-axis represents the average fate of the alleles (replicate of 10,000 with a starting population of 10,000) from the given starting frequency shown in Figure 5 for each of the lines.

If resistance to the first factor is recessive, the first NCR toxin is used on its own, until the resistance allele becomes common enough in the population that the first toxin is no longer effective in controlling the population. At this time, the toxin pair is then used on the insect population such that the allelic frequency of resistance moves back to a low level. Using the second NCR factors sparingly may ultimately be more economically acceptable than the continuous use of two toxins. Thus, as seen from the preceding Figures, resistant alleles can be managed in a target population.

Screening for Negative Cross-Resistance Factors

The current generation of pesticides includes toxins isolated from bacterial broths, such as Spinosad, and transgenic plants containing genes that code for an insecticidal protein. It is highly likely that in some cases target-site insensitivity to these new classes of insecticides occur in the pest insects. Target-site insensitivity is a major mechanism of resistance to second generation pesticides. After deploying these novel toxins, it is likely that a single (or multiple) point mutation in the gene coding for the target site in the insect's gut or other target system results in the insects developing field resistance. Additionally, metabolic resistance may occur where the insects have a greater ability to alter the toxin such that it has reduced toxic activity. Even if metabolic resistance occurs to such resistance factors, the metabolic resistance does not rule out the possibility of developing NCR compounds for control of metabolic insecticide resistance.

Modern pesticide and antibiotic discovery involves the routine (and often automated) screening of tens or hundreds of thousands of candidate toxins against a repertoire of insects (and bacteria in the case of antibiotic discovery). Once a toxin has been deployed, and resistance arises, there have been few cases where researchers have made an effort to determine the existence of NCR factors.

In spite of the lack of large-scale screening for NCR toxins, there still has been discovery of such compounds. For example, it has been found that AaIT, a protein isolated from Scorpion toxin, provides NCR to pyrethroids in knockdown resistance *(kdr)* files. In addition, a NCR factor to aphids has been identified that was resistant to insecticides through increased production of a carboxylesterase, E4.

Although NCR factors do occur within classes of toxins, there is no distinct reason to believe that NCR factors will only be found in the same class of compounds as the first toxin. Although compounds within the classes of toxins appear to be a logical starting place, exemplary screens for NCR toxins involve random screens for compounds. The random screens are, in one embodiment, coupled with a 'clue-based' screen.

Method of Screening for Negative Cross-Resistance Factors

An advantage of random screening for NCR factors is that an understanding of the molecular basis of resistance is not necessary for the development of the second compound. Knowledge on the molecular basis of resistance typically lags years behind the first appearance of resistant insects in the field. However, knowing the basis of resistance is helpful for 'clue-based' screening. But if discovery of the molecular basis of pesticide resistance is too costly or time consuming, one may be able to use the resistant line (or lines) in a random screen for NCR factors.

Tests using resistant and susceptible lines of insects are easily integrated into current large-scale automated screening methodologies. The screens identify compounds that are toxic to the resistant line (or lines) in the bioassay and not toxic to the insect lines that are susceptible to the already commercialized toxin.

Figure 6 illustrates a method 100 of evaluating the efficacy of compounds to be deployed commercially as NCR factors. A first toxin is developed and deployed 102 commercially. The first toxin is lethal to at least a portion of a target population, e.g., an insect population. A susceptible strain (S/S) of the target population is determined that is susceptible to the first toxin. After the first toxin has

been used for a period of time, a strain of the target population that is resistant to the first toxin begins to develop and grow. The resistant strain is selected for 104 to be used in a NCR evaluation. In one embodiment, the resistant strain is selected for using a field collected strain. In an alternative embodiment, the resistant strain is selected for using an EMS-mutagenized line selected for pesticide resistance. One of the disadvantages of selection through EMS-mutagenized lines is that mutants may be found that confer resistance to the toxin, but the alleles may not be commonly observed in nature.

For example, DDT and pyrethroid resistance mutations have been identified in regions of the *Drosophila* sodium channel, *para*, that have not been observed in nature. One of the resistance mutation lines studied showed no NCR to AaIT, but the naturally occurring *kdr* (from field lines of insects) showed 9 to 14-fold more susceptibility to this compound. Thus, the use of EMS-mutagenized lines to screen for negative cross resistance in large-scale bioassays may result in failing to identify compounds that may be effective in resistance found in the field. Alternatively, one may also identify NCR factors from EMS-mutagenized lines that may not be ultimately useful in field resistant lines of insects.

After the resistant strain has been selected for, a homozygous resistant strain (R/R) is evaluated 106 with a number of potentially toxic molecules, e.g., natural molecules, synthetic molecules, chemicals, compounds, biotechnical species, and biotechnical moieties, to determine a second toxin that is more toxic to the resistant strain (R/R) than to the susceptible strain (S/S). In one embodiment, the toxic molecules include variants, mutants, metabolites, and derivatives.

In addition, a susceptible control strain (S/S) is also evaluated with the same compounds. In one embodiment, the strains are evaluated with between about 10 and 10^9 compounds. In an alternative embodiment, the strains are evaluated with about 10^2 to about 10^8 compounds. In a further alternative embodiment, the compounds are evaluated with about 10^3 to about 10^7 compounds. In one embodiment, compounds to be screened include chemicals from known pesticides, insect biocides, and their variants, mutants, metabolites, and derivatives. Exemplary

chemicals include a) Bacillus thuringiensis proteins and their variants, b) clorinated hydrocarbons, c) organophosphates, d) pyrethroids, e) carbamates, f) variants of toxins from the bacteria Photorhabdus luminescens, g) insect growth regulators and their derivatives, h) alpha-amylase inhibitors, i) lectins, j) Spinosad derivatives, k) spinosyns and their derivatives, l) derivatives of insecticidal compounds from the bacteria Saccharopolyspora spinosa, m) Bacillus thuringiensis strains and their variants, n) protease inhibitors and their derivatives, o) Cysteine protease inhibitors and their derivatives, p) Bowman-Birk Inhibitors and their derivatives, q) Kunitz inhibitors and their derivatives, r) Saccharopolyspora spinosa strains and derivatives of their insecticidal and non-insecticidal toxins, and s) imidacloprid or derivatives of imidacloprid.

In a further embodiment, molecules are supplied from randomly or selectively generated chemicals, and random or selective (chemical rationale approach) screening of chemicals. The molecules to be evaluated further include molecules supplied from bio-prospecting from plant, animal, bacteria, and fungal organisms or extracts of these organisms and from prokaryotic or eukaryotic organisms. The molecules to be evaluated also include molecules supplied from the generation of antibodies showing preference for binding to proteins or protein complexes or membranes in the organism involved in negative cross resistance (binding preference for versions of the protein that are resistant to the first toxin) and generation of random peptide libraries and bio-panning using phage display. A random peptide library is made and is screened for affinity to the product of the target of interest, e.g., the gene product of the target site. The resistant allele, more specifically the protein product, is then used to identify a protein that has high affinity to the gene product to generate a NCR toxin for specifically targeting the resistant insect. The molecules to be evaluated also include molecules obtained from combinatorial shape libraries and molecules supplied using combinatorial chemistry.

The above described compounds are exemplary only and are not intended to limit the compounds to only those described above. In addition, although method 100 describes evaluations, it should be understood by one of ordinary skill in the art that screening methods can be utilized for the evaluations.

Those compounds that are more toxic to the resistant strain than to the susceptible strain are considered to be positive compounds for the initial evaluation. A heterozygous strain (R/S) of the target population is evaluated 108 with the positive compounds to test their effectiveness against the heterozygous insects. Thus, the resistant (R/R) and susceptible (S/S) insects are crossed and the progeny bio-assayed against the new toxin. It should be determined whether resistance is sex-linked, since if the resistance is sex-linked, individuals of the proper sex that carry two alleles of the gene should be used. For example, since Drosophila is XY for males and XX for females, a bioassay in this species for a sex linked resistance should initially focus on females. The heterozygotes are screened by using separate applications of the first toxin and the positive compound being tested, i.e., the second toxin, to determine if separate applications of the first toxin and the second toxin are at least as toxic to the heterozygous strain (R/S) as to the susceptible strain (S/S) of the target population.

If the heterozygotes are killed by separate applications of the first toxin and the positive compound being tested 110, the positive compound is given a high priority for development 112 and commercial exploitation. A high negative cross resistance priority is assigned to the second toxin if separate applications of the first toxin and the second toxin are at least as toxic to the heterozygous strain (R/S) as to the susceptible strain (S/S). Thus, based on the toxicity of the compound to heterozygous individuals, the practical applicability of each toxin is prioritized and the compounds capable of killing the heterozygotes receive a high priority while those compounds that only impact homozygous individuals are subjected to further testing and evaluation to determine their prioritization. The priority compounds, in one embodiment, are prioritized for advancement to additional evaluations which are utilized to make commercial development prioritization decisions. In an alternative embodiment, the high priority compounds receive a commercialization prioritization.

The heterozygous strain is evaluated 114 with the first toxin and the second toxin applied at the same time to determine 116 if the application of the first toxin and the second toxin at the same time is at least as toxic to the heterozygous strain (R/S) as to the susceptible strain (S/S). If the application of the first toxin and the second toxin at the same time is not at least as toxic to the heterozygous strain as

to the susceptible strain 116, the compound is given a low priority for further development.

In an alternative embodiment, the second toxins are prioritized based on their performance in the resistant strain evaluation and in the heterozygous strain evaluation. At least one of the highest prioritized second toxins is selected for advancement to additional evaluation to determine a commercialization prioritization.

If the application of the first toxin and the second toxin at the same time are at least as toxic to the heterozygous strain as to the susceptible strain 116, then a determination is made regarding whether both compounds can be applied 120 at the same time at an economically acceptable rate. If both compounds cannot be applied at the same time at an economically acceptable rate, the compounds should be given a low priority 122 for further development. If both compounds can be applied at the same time at an economically acceptable rate, the compound should be considered 124 for commercial development. For example, an economically acceptable rate is, in one embodiment, a rate that someone is willing to pay for using the compounds to obtain a desired effect on the target population.

In one embodiment, a high negative cross resistance priority is assigned to the second toxin if the first toxin and the second toxin can be applied to the target population at the same time at an economically acceptable rate. For compounds that test positive for application at the same time, the second toxin, in one embodiment, is applied every time the first toxin is applied. In an alternative embodiment, the second toxin is applied intermittently with application of the first toxin, e.g., the second toxin is applied every other time the first toxin is applied.

Thus it can be seen that once a pair of NCR factors is determined, many different types of applications of the toxins to the insects can be used. For example, both toxins can be applied at the same time every time, one of the toxins can be applied on an intermittent basis, both toxins can be applied on an intermittent basis, and the toxins can be applied in an alternating type application. In one embodiment, the toxins are delivered to the target population utilizing at least one of sprays, pellets, powders, baited or non-baited traps, and transgenic organisms. For example, in the

case of weeds, a first compound is applied to the field by spraying the compound on the weeds. If resistant forms exist in the particular field, a second compound is then applied to the field. In addition, transgenic antibodies or antibody conjugates with toxins attached could be used in the selection assays. Thus, in one embodiment, the above described method is used to manage a tract of land against a resistant strain of a target population. In an alternative embodiment, the above described method is used to control an insect population on a tract of land. In a further alternative embodiment, the above described method is used as part of a pest management system to manage a pest population.

Although the evaluations described above are described in the context of whole organism screens, the above described method is not limited to whole organism screening. For example, components from the insects could be used for adaptations in *in vitro* screens of target sites. It is known that insects develop resistance to plant inhibitors and bio-panning helps to develop new forms of inhibitors useful in control of the insects.

In an alternative embodiment, a compound is selected *in vitro* that works better at the resistant target site than the susceptible target site. This compound is then subjected to the screening process described above to determine if the compound is a viable NCR factor.

In a further alternative embodiment, the above described method is utilized to select for a first virus that kills the insects resistant to a second virus. The viruses are made transgenic in plants. Eventually, the insects become resistant to the first virus. The above method is then used to evaluate variants of the viruses or other related viruses.

Another form of negative cross-resistance is exemplified with DDT resistance in Drosophila melanogaster (Drosophila) and the compound phenyl-thio-urea (PTU). When PTU is fed to metabolically resistant Drosophila, the cytochrome P450 enzymes causing resistance to DDT bio-activate the PTU into a toxic compound. The DDT-resistant Drosophila line has higher mortality and takes longer to develop than the susceptible flies. In an alternative embodiment of the invention,

potential NCR compounds are screened to determine if the toxins impact other life-history parameters, e.g., delays in developmental time, reduction of the fecundity of only the resistant insect lines. If a NCR toxin is used that delays developmental time, the resistance allele can be kept at a lower level in the population.

Exemplary insect lines that can be used to screen for compounds include, but are not limited to, Drosophila lines including Rst(2)DDT-Wisconsin and Rst(2)DDT-Hikone. Both lines exhibit NCR between DDT and PTU. More particularly, Rst(2)DDT (also known as Dimethylnitrosamine demethylase; Dmnd) exists between cinnabar (cn) and vestigal (vg) and evidence exists to suggest that the Rst(2)DDT locus is the gene CYP6G1 (a cytochrome P450 gene) and resistance is due to over-expression in the Rst(2)DDT-Wisconsin fly line. For example, Rst(2)DDT is located at the cytological position 48A-49D (Recombination map position 64.5-66.0 on Chromosome 2). The position of CYP6G1 is 48F1(Cytological position) and it thus lies in the middle of the resistance region. See for example, FlyBase (http://cbbridges.harvard.edu:7081/).

Figure 7 is a Northern blot of Canton-S (Can-S), DDT-resistant line with no exposure to DDT (Rst(2)DDT-Wisconsin), and the DDT-resistant fly line with exposure to 20 μg /vial of DDT for 24 hours (Rst(2)DDT-Wisconsin–20 μg of DDT). The blot was probed with actin and CYP6G1 cDNA.

Total RNA was extracted from the flies, and run on a 1.5% agarose gel for 3.5 hours. The RNA was transferred to a nylon membrane, and the membrane was probed with P32 labeled gene fragments in a hybridization solution overnight. The blots were washed in a 2 X SSC (+ 0.1% SDS) solution three times at 15 minutes per wash and in a 2 X SSC (+ 0.1% SDS) solution three times at 15 minutes per wash. The blots were then exposed to X-ray film for a time period that clearly showed the bands with the minimal amount of background.

Resistance has been associated with increased expression of cytochrome P450 genes, as is typical of metabolically resistant insects. In the resistant line, over-expression of CYP6G1, which had >10-fold expression as compared to the susceptible fly line (Canton-s), was observed. Evidence exists to suggest that the

Rst(2)DDT-Wisconsin flies dramatically over-express resistance. Resistance may be due to at least one of over-expression in the resistance line (genotype) and over-expression and a mutation in the CYP6G1 gene in the resistance line (genotype).

In one embodiment of the invention, the above described method is utilized with an Rst(2)DDT fly line to develop chemicals that selectively target resistance. If this mechanism of resistance is common to other species, metabolically resistant Drosophila can be used to screen for NCR toxins that can reduce metabolic resistance in other insect species. In an exemplary embodiment, the screening method is used to identify toxins to minimize metabolic resistance in mosquitoes that transmit malaria. Rst(2)DDT is known to confer resistance to DDT, chlordane, lindane, and imidacloprid and Rst(2)DDT can be targeted to develop toxins to control metabolic resistance to one or all of these compounds.

In alternative embodiments, the above described methods are utilized with putative target sites other than Rst(2)DDT. Exemplary putative target sites include, but are not limited to, acetylcholinesterases, voltage-gated sodium channels, GABA receptors (Rdl or Resistance to Dieldrin in Drosophila is an example), esterases, cytochrome P450s, neurotransmitter uptake channels, cation and ion channel, aromatic biogenic amine receptors, and Glutathione-S-Transferases. Anther putative target site is CY6G1 in Drosophila also currently refereed to as to as (a) CG8453, (b) AF083946, C6G1_DROME, AC Q9V674 and O76800 in gene nomenclature.

Several resistant lines, with potentially different forms of resistance can be incorporated into the bio-assays. Thus screens can be conducted for a series of toxins before resistance becomes problematic in the field, and when resistance occurs in the field, at low frequency, this repertoire of toxins can be tested against the emerging resistant lines. Other management strategies can be incorporated into field practices to slow the rate of entry of these resistance alleles into the population while there is time to develop the NCR factor to a commercial level.

Figure 8 is a method 150 of providing management decisions for the development of NCR factors. Method 150 includes observing 152 field resistance

against a compound commercially deployed to reduce the number of pests in a target population on a tract of land. Testing 154 is conducted on a group of NCR factors that were discovered to be effective against the resistant lines bio-assayed in the laboratory. The testing is conducted against the field resistant line of the target population. A determination is made 156 regarding whether any compound tested is effective against the target population. If no compounds are found to be effective against the target population, the large scale screening process is repeated 158 using field resistant insects in the bioassay. If compounds are found to be effective against the target population, the effective compounds are prioritized 160 and a decision is made for each such compound regarding whether to initiate 162 a resistant management program to slow entry of the resistant alleles into the insect population and commercially develop 164 the compound.

A first factor influencing the decision to develop a NCR factor is the diversity of resistance mechanisms in the field. If the forms of resistance that occur in the field are highly uniform (i.e., similar forms of resistance) this uniformity increases the priority given to the development of negative-cross resistance factors. Commercial development of a NCR factor may be feasible if there are highly uniform amino acid changes across divergent taxa and the resistance mechanism is uniform in the field. However, if there is a great diversity in nature regarding how insects develop resistance to a commercially deployed pesticide, it may be difficult to identify a single compound that provides generalized NCR. If the resistance shows diversity, development of a single NCR factor may be expensive and multiple NCR compounds may prove too costly for combating resistance.

Economics is the second factor influencing the decision to develop a NCR factor. Resistance to toxins that have little commercial value, such as insecticides that are useful in minor or niche markets, may not justify the costs of developing NCR factors. Alternatively, resistance to commonly used antibiotics may warrant the development of multiple NCR factors (effective against different forms of resistance) due to the commercial value of these compounds.

Screens for NCR factors may also be useful in the discovery of compounds that kill antibiotic resistant bacteria. Large-scale screens can be performed with antibiotic susceptible and resistant bacterial strains to identify compounds that selectively kill the antibiotic resistant (but not the susceptible) bacteria. These compounds can then be incorporated into materials or medicines that reduce the numbers of antibiotic resistant bacteria in a given environment. As with pesticide resistance, the diversity of resistance alleles and the nature of the antibiotic resistance genes influence how feasible such a screen is for dealing with antibiotic resistance.

For example, after performing method 100 (shown in Figure 6) on a compound, and prior to commercial development of the compound, testing is conducted to determine how useful the compound would be in the field. Field trials of the compound involve testing a field including a first organism, e.g., an insect, plant, bacteria, virus, and others, with a mixture of resistant and susceptible lines (genotypes/phenotypes). The frequency of resistance is determined before application and after application to determine an effectiveness of the compounds. Thus, the above described methods provide for screening a compound, such as in a lab, determining a potentially beneficial compound, and testing the potentially beneficial compound in the field.

Implementation of Negative Cross-Resistance Factors

The development and effective deployment of NCR factors utilize a combination of basic research, marketing, extension services, and field applicators of the product. In one embodiment, research teams access field collected lines of resistant insects to develop effective toxins capable of killing insects that contain those alleles thought most likely to emerge in a field population. Once basic research programs determine the underlying molecular basis of field resistance this information is used to develop molecular diagnostics to determine the frequency of the allele in the insect population being targeted.

Marketing programs along with extension services provide feedback on the allelic frequency of resistance in the field. Decisions regarding when to begin to

use the second NCR factor are made once the allelic frequency of resistance in the field is known. If monitoring programs are too costly or ineffective, treatment of the target population early in the growing season to reduce the resistance allele in the population is sometimes an effective way to minimize the development of a resistant insect population throughout the field season. In one embodiment, the NCR factors are delivered to the target population utilizing at least one of sprays, pellets, powders, baited or non-baited traps, and transgenic organisms.

Experiments

A model for the developmental cycle of a hypothetical insect pest was formulated and the following assumptions were made. The number of progeny per female had a Poisson distribution, mean fecundity, μ and was assumed to be 60 offspring per female. When there were fewer than 20 mating pairs the progeny was simulated directly. Above 20 mating pairs, the total number of progeny was approximated using the normal distribution. The number of insects of each genotype among the total progeny has a multinomial distribution with the proportion of each genotype being given by their respective Hardy-Weinberg frequencies. The number of males and females was binomially distributed with the expected ratio of males to females in the population being 1:1. The proportion of mutant alleles in the population is initially equal to m (mutation rate). Initially there are N_0 insects in different stages of development in the population. Each insect mates once on average. Mating occurs, with equal probability, at any time during the breeding period of the insect's adulthood (3.5 days of breeding). Mutation rate (per individual, per unit time) is constant, as is female fecundity. The developmental times for the following stages are egg (7 days), larval development (30 days) and reproductive period (3.5 days). The standard (background) mortality rate was calculated using an egg mortality of 5%, a larval establishment mortality of 5%, and a larval developmental mortality of 20%. It is further assumed that the genotype does not affect the background mortality rate such that the background mortality is independent of the mortality caused by the pesticide or pesticides.

A Monte Carlo simulation tool, i.e., a decisioneering tool, was used to estimate the maximum population size and mutant allelic frequency after a given number of days. The model calculated these values at each half-day. Given the number of adults mating during a time interval, the number of progeny was determined, and these progeny were classified as homozygous wild-type, homozygous mutants, or heterozygous for these alleles (based on Hardy-Weinberg frequencies). Selection and mutation occurred before the mating phase for each insect.

As expected, if the two toxins did not cause equal mortality rates to the respective homozygous insects, the alleles of the fitter homozygotes became more common over time. In all but one of the cases examined, the population size was below the starting level of 10,000 individuals when the fitter allele reached a frequency of 50%. As the allelic frequency of the fitter allele increased beyond 50% the population grew rapidly. The closer the two NCR compounds were in toxicity, the longer it took for the fitter allele to increase in frequency. High toxicity of both compounds to homozygotes and heterozygotes resulted in effective control of the insect population.

When the combined toxins impacted the heterozygotes less than the homozygotes, the allelic frequency of the resistance allele approached 50% asympotically. The greater the difference in mortality rates between the heterozygotes and homozygotes the faster the resistance allele approached 50% frequency. But if the combined toxins killed more heterozygotes than homozygotes and the resistance allele was rare at the start of the experiment, the allele was likely to remain rare.

If the resistance allele was already common in the population when the two-toxin regime (both compounds used at once) was initiated (where there was greater heterozygote toxicity than homozygote toxicity), the resistance allele ultimately went to an extreme value (near 0% or near 100%). The probability of the allele going to fixation (100%) or extinction (0%), or close to these values, depended on its starting frequency. If the resistance allele started below 50%, on average it would tend towards extinction. The further the starting allelic frequency was below 50% the greater the probability that the allele would be lost from the population. On

the other hand, if the frequency of the resistance allele exceeded 50% at the start of the concurrent treatments, then the resistance allele would on average go to fixation. If the starting allelic frequency was at 50% then the allele was as likely to tend towards fixation as it was to go to extinction.

Simulations were also performed in which both NCR factors were applied together and their toxicity was varied to determine which conditions provided for the most effective control of the insect population. Four specific cases were investigated. First, the mortality rate of one homozygote group was 99% and the mortality rates of the heterozygotes and the other group of homozygotes was varied. Second, the effect of varying heterozygous fitness was examined when the mortality rates of the two homozygous groups were equal. Third, the impact of starting allelic frequency on the fate of the alleles was reviewed when the two toxins killed the heterozygotes at a higher rate than the homozygotes. Fourth, the second toxin was used intermittently to minimize the frequency of the resistance allele.

Example

A test was conducted utilizing an initial screen of DDT and 8 pyrethroids against Canton-S (DDT susceptible flies) and *para*^{tsl} (DDT resistant flies). Control flies, flies not exposed to toxins, were also screened and showed no mortality. Table 3 illustrates the results of this initial screen.

Table 3

Compound	Fly Line Preferentially Killed by the Toxin	Experiment-wise P-Value
DDT	Canton-S	0.039
Deltamethrin	*Para*^{tsl}	0.0156
D-trans allethrin	Neither	>0.20
Tempo	*Para*^{tsl}	0.125
Asana	Neither	>0.20
Resmethrin	*Para*^{tsl}	>0.20
PP321	Neither	>0.20
Permethrin	*Para*^{tsl}	0.039
PP993	Canton-S	0.18

As seen in Table 3, across the doses, deltamethrin and permethrin were more toxic to *para*^{tsl} flies than to Canton-S flies.

Table 4 illustrates the mortality of Canton-S flies and *para*^{tsl} flies in the presence of either DDT or deltamethrin. Control flies showed no mortality.

Table 4

Compound	Dose	Percent Dead		P-Values	
		Cantons-S	*Para*^{tsl}	Non-Corrected	Corrected
DDT	20	71.7	6.7	0.012	0.084
	15	75.0	8.3	0.0059	0.041
	10	43.3	13.3	*	*
	8	48.3	23.3	*	*
	5	35.0	6.7	*	*
	2	15.0	3.3	*	*
	1	6.7	0	*	*
	0.5	8.3	0	*	*
	0.2	11.7	13.3	*	*
Deltamethrin	20	98.3	100	*	*
	15	100	100	*	*
	10	98.3	98.3	*	*
	8	96.7	100	*	*
	5	100	100	*	*
	2	93.3	100	*	*
	1	26.7	85.0	0.0128	0.102
	0.5	56.7	100	*	*
	0.2	31.7	98.3	0.0037	0.030
* P>0.20					

As illustrated in Table 4, when tested by individual doses, only deltamethrin preferentially killed *para*^{tsl} flies. The other pyrethroids tested did not show significantly different toxicity between Canton-S flies and *para*^{tsl} flies at any of the specific doses.

As a further step in the test, heterozygotes were tested with either deltamethrin or DDT. The effect of deltamethrin on Canton-S flies, *para*^{tsl} flies, and heterozygous fly lines is illustrated in Table 5.

Table 5

Fly Line	Male/Female	LD_{50}	95% CI	N	Resistance Ratio
Canton-S	Female	0.988	0.075-2.22	830	24.7
	Male	0.396	0.287-0.495	420	9.9
para^{tsl}	Female	0.057	0.043-0.074	500	1.4

	Male	0.040	0.025-0.057	520	1.0*
Heterozygotes	Female	0.860	0.652-1.128	830	21.5
* The resistance ratio is defined as the respective fly line LD_{50} as the numerator and the LD_{50} of the *para*^{tsl} males as the denominator.					

As seen in Table 5, heterozygotes are resistant to deltamethrin and resistance is dominant. In addition, it is known that heterozygotes are resistant to DDT. Thus it was determined that resistance was not recessive to either deltamethrin or DDT. An additional test was conducted to determine if DDT and deltamethrin applied at the same time killed the heterozygotes.

Figure 9 graphically illustrates the application of deltamethrin by itself 200, the application of DDT by itself 202, and the application of both deltamethrin and DDT at the same time 204 to *para*^{tsl} flies, Canton-S flies, and heterozygotes. As illustrated in Figure 8, both deltamethrin and DDT together killed the heterozygotes better than either deltamethrin or DDT on its own.. Therefore, DDT and deltamethrin combined effectively to kill the heterozygotes and it was determined that deltamethrin and DDT are NCR factors.

While the invention has been described in terms of various specific embodiments, those skilled in the art will recognize that the invention can be practiced with modification within the spirit and scope of the claims.

WHAT IS CLAIMED IS:

1. A method of evaluating the efficacy of a molecule against a target population, the target population including a strain resistant to a first toxin, said method comprising:

determining a susceptible strain in the target population, the susceptible strain being susceptible to the first toxin;

selecting for the resistant strain in the target population, the resistant strain being resistant to the first toxin;

evaluating the efficacy of the resistant strain with a plurality of molecules to determine a second toxin that is more toxic to the resistant strain than to the susceptible strain;

evaluating the efficacy of a heterozygous strain of the target population with separate applications of the first toxin and the second toxin; and

assigning a priority rating to the second toxin if the separate applications of the first toxin and the second toxin are at least as toxic to the heterozygous strain as to the susceptible strain.

2. A method in accordance with Claim 1 further comprising screening the heterozygous strain with the first toxin and the second toxin applied at the same time.

3. A method in accordance with Claim 2 further comprising assigning a priority rating to the second toxin if the application of the first toxin and the second toxin at the same time are at least as toxic to the heterozygous strain as to the susceptible strain.

4. A method in accordance with Claim 1 further comprising determining whether both the first toxin and the second toxin can be applied to the target population at the same time at an acceptable rate.

5. A method in accordance with Claim 4 further comprising assigning a priority rating to the second toxin if the first toxin and the second toxin can be applied to the target population at the same time at an acceptable rate.

6. A method in accordance with Claim 1 wherein the target population is an insect population.

7. A method in accordance with Claim 1 wherein selecting for the resistant strain in the target population comprises selecting for a homozygous resistant strain in the target population.

8. A method in accordance with Claim 1 wherein selecting for the resistant strain in the target population comprises selecting for a resistant strain in the target population using at least one of a field collected line and an EMS-mutagenized line.

9. A method in accordance with Claim 1 wherein evaluating the efficacy of the resistant strain comprises evaluating the efficacy of the resistant strain with between about 10 and 10^9 molecules.

10. A method of testing for negative cross resistance in a target population, said method comprising:

determining a susceptible strain (S/S) in the target population, the susceptible strain (S/S) susceptible to a first toxin;

selecting for a resistant strain (R/R) in the target population, the resistant strain (R/R) resistant to the first toxin;

evaluating the efficacy of the resistant strain (R/R) with between about 10 and 10^9 molecules to determine a second toxin that is more toxic to the resistant strain (R/R) than to the susceptible strain (S/S);

evaluating the efficacy of a heterozygous strain (R/S) of the target population with separate applications of the first toxin and the second toxin to

determine if separate applications of the first toxin and the second toxin are at least as toxic to the heterozygous strain (R/S) as to the susceptible strain (S/S); and

assigning a high negative cross resistance priority to the second toxin if the separate applications of the first toxin and the second toxin are at least as toxic to the heterozygous strain (R/S) as to the susceptible strain (S/S).

11. A method in accordance with Claim 10 further comprising evaluating the efficacy of the heterozygous strain (R/S) with the first toxin and the second toxin applied at the same time to determine if the application of the first toxin and the second toxin at the same time is at least as toxic to the heterozygous strain (R/S) as to the susceptible strain (S/S).

12. A method in accordance with Claim 11 further comprising determining whether both the first toxin and the second toxin can be applied to the target population at the same time at an economically acceptable rate.

13. A method in accordance with Claim 12 further comprising assigning a high negative cross resistance priority to the second toxin if the first toxin and the second toxin can be applied to the target population at the same time at an economically acceptable rate.

14. A method in accordance with Claim 10 wherein the target population is an insect population.

15. A method in accordance with Claim 10 wherein selecting for a resistant strain (R/R) in the target population comprises selecting for a resistant strain (R/R) in the target population using at least one of a field collected line and an EMS-mutagenized line.

16. A method of screening chemicals to determine a commercialization prioritization for the compounds, said method comprising:

determining a susceptible strain (S/S) in a target population, the susceptible strain (S/S) susceptible to a first toxin;

selecting for a resistant strain (R/R) in the target population, the resistant strain (R/R) resistant to the first toxin;

testing the resistant strain (R/R) with a number of molecules to determine at least one chemical that is more toxic to the resistant strain (R/R) than to the susceptible strain (S/S);

testing a heterozygous strain (R/S) of the target population with separate applications of the at least one chemical and the first toxin to determine if the at least one chemical and the first toxin are at least as toxic to the heterozygous strain (R/S) as to the susceptible strain (S/S);

assigning a high priority to the at least one molecule if the at least one molecule and the first toxin are at least as toxic to the heterozygous strain (R/S) as to the susceptible strain (S/S);

testing the heterozygous strain (R/S) with both of the at least one molecule and the first toxin applied at the same time to determine if the combination of the at least one molecule and the first toxin is at least as toxic to the heterozygous strain (R/S) as to the susceptible strain;

determining whether both the at least one molecule and the first toxin can be applied to the target population at the same time at an acceptable rate; and

assigning a high priority to the at least one molecule if the at least one molecule and the first toxin can be applied to the target population at the same time at an economical rate.

17. A method in accordance with Claim 16 wherein selecting for a resistant strain (R/R) in the target population comprises selecting for a resistant strain (R/R) in the target population using at least one of a field collected line and an EMS-mutagenized line.

18. A method in accordance with Claim 16 wherein screening the resistant strain (R/R) comprises screening the resistant strain (R/R) with between about 10 and 10^9 molecules.

19. A method in accordance with Claim 16 wherein the target population is an insect population.

20. A method for managing resistant alleles in a target population, said method comprising:

determining a susceptible strain in the target population, the susceptible strain being susceptible to the first toxin;

selecting for a resistant strain in the target population, the resistant strain including at least one resistant allele, the resistant allele providing resistance to the resistant strain against the first toxin;

screening the resistant strain with a plurality of molecules to determine a second toxin that is more toxic to the resistant strain than to the susceptible strain;

screening a heterozygous strain of the target population with separate applications of the first toxin and the second toxin; and

prioritizing the second toxin for commercialization testing if the separate applications of the first toxin and the second toxin are at least as toxic to the heterozygous strain as to the susceptible strain.

21. A method in accordance with Claim 20 further comprising:

screening the heterozygous strain with the first toxin and the second toxin applied at the same time; and

prioritizing the second toxin for commercialization testing if the application of the first toxin and the second toxin at the same time are at least as toxic to the heterozygous strain as to the susceptible strain.

22. A method in accordance with Claim 20 further comprising:

determining whether both the first toxin and the second toxin can be applied to the target population at the same time at an economical rate; and

prioritizing the second toxin for advancement to additional screening if the first toxin and the second toxin can be applied to the target population at the same time at an economically acceptable rate.

23. A method of managing a tract of land against a resistant strain of a target population, said method comprising:

determining a susceptible strain in a target population, the susceptible strain susceptible to the first toxin;

selecting for the resistant strain which is resistant to the first toxin;

evaluating the resistant strain to determine a second toxin that is more toxic to the resistant strain than to the susceptible strain;

evaluating a heterozygous strain of the target population with separate applications of the first toxin and the second toxin; and

utilizing the second toxin to manage the land against the resistant strain if the separate applications of the first toxin and the second toxin are at least as toxic to the heterozygous strain as to the susceptible strain.

24. A method in accordance with Claim 23 further comprising:

evaluating the heterozygous strain with the first toxin and the second toxin applied at the same time; and

utilizing the second toxin to manage the land against the resistant strain if the application of the first toxin and the second toxin at the same time are at least as toxic to the heterozygous strain as to the susceptible strain.

25. A method of managing a portfolio that includes a plurality of molecules, said method comprising:

selecting a target population;

obtaining a first strain in the target population, the first strain susceptible to a first toxin;

obtaining a second strain in the target population, the second strain resistant to the first toxin;

evaluating the efficacy of the second strain with multiple molecules in the portfolio to determine multiple second toxins that are more toxic to the second strain than to the first strain;

evaluating the efficacy of a third strain of the target population with separate applications of the first toxin and the second toxins, the third strain heterozygous for resistance to the first toxin; and

prioritizing the second toxins based on their performance in the second strain evaluation and in the third strain evaluation.

26. A method in accordance with Claim 25 further comprising selecting the highest prioritized second toxin for advancement to additional evaluation to determine a commercialization prioritization.

27. A method in accordance with Claim 25 further comprising:

evaluating the efficacy of the third strain with the first toxin and the second toxin applied at the same time; and

selecting the second toxin for advancement to additional evaluation to determine a commercialization prioritization if the application of the first toxin and the second toxin at the same time are at least as toxic to the third strain as to the first strain.

28. A method in accordance with Claim 25 further comprising:

determining whether both the first toxin and the second toxin can be applied to the target population at the same time at an acceptable rate; and

selecting the second toxin for advancement to additional evaluation to determine a commercialization prioritization if the first toxin and the second toxin can be applied to the target population at the same time at an acceptable rate.

29. A method for assessing whether to develop a molecule included within a molecule portfolio, said method comprising:

evaluating the efficacy of a resistant strain of a target population with molecules in the molecule portfolio;

selecting a molecule that is more toxic to the resistant strain than to a susceptible strain, the susceptible strain susceptible to a first toxin, the resistant strain resistant to the first toxin;

evaluating the efficacy of a heterozygous strain of the target population with separate applications of the first toxin and the selected molecule, the heterozygous strain heterozygous for resistance to the first toxin; and

selecting the molecule for commercialization if the separate applications of the first toxin and the selected molecule are at least as toxic to the heterozygous strain as to the susceptible strain.

30. A method in accordance with Claim 29 further comprising:

evaluating the efficacy of the heterozygous strain with the first toxin and the selected molecule applied at the same time; and

selecting the selected molecule for a commercialization track if the application of the first toxin and the product at the same time are at least as toxic to the heterozygous strain as to the susceptible strain.

31. A method in accordance with Claim 29 further comprising:

determining whether the first toxin and the selected molecule can be applied to the target population at the same time at an acceptable rate; and

selecting the selected molecule for a commercialization track if the first toxin and the selected molecule can be applied to the target population at the same time at an economically acceptable rate.

32. A method in accordance with Claim 29 wherein the molecule portfolio includes chemicals and evaluating the efficacy of a resistant strain of a target population with molecules in the molecule portfolio comprises evaluating the efficacy of a resistant strain of a target population with chemicals in the chemical portfolio.

33. A method in accordance with Claim 29 wherein the molecule portfolio includes compounds and evaluating the efficacy of a resistant strain of a target population with molecules in the molecule portfolio comprises evaluating the efficacy of a resistant strain of a target population with compounds in the compound portfolio.

34. A method for controlling an insect population on a tract of land, said method comprising:

obtaining an insect strain susceptible to a first toxin;

obtaining an insect strain resistant to the first toxin;

screening the resistant insect strain with a plurality of molecules;

determining a second toxin that is more toxic to the resistant insect strain than to the susceptible insect strain;

screening a heterozygous insect strain with separate applications of the first toxin and the second toxin;

determining if separate applications of the first toxin and the second toxin are at least as toxic to the heterozygous strain as to the susceptible strain; and

utilizing the second toxin to control the insect population on the tract of land if separate applications of the first toxin and the second toxin are at least as toxic to the heterozygous insect strain as to the susceptible insect strain.

35. A method in accordance with Claim 34 further comprising:

screening the heterozygous strain with the first toxin and the second toxin applied at the same time: and

determining if the application of the first toxin and the second toxin at the same time is at least as toxic to the heterozygous strain as to the susceptible strain.

36. A method in accordance with Claim 35 further comprising:

determining whether the first toxin and the second toxin can be applied to the target population at the same time at an acceptable rate; and

utilizing the second toxin to control the insect population if the first toxin and the second toxin can be applied to the target population at the same time at an acceptable rate.

37. A method for utilizing a pest management system to manage a pest population, the system including a plurality of molecules, said method comprising:

determining a susceptible strain in the pest population, the susceptible strain susceptible to a first toxin;

selecting for a resistant strain in the pest population, the resistant strain resistant to the first toxin;

evaluating the resistant strain to determine a second toxin that is more toxic to the resistant strain than to the susceptible strain;

evaluating a heterozygous strain with the first toxin and the second toxin applied at the same time; and

utilizing the second toxin to manage the pest population if the application of the first toxin and the second toxin at the same time are at least as toxic to the heterozygous strain as to the susceptible strain.

38. A method in accordance with Claim 37 further comprising:

evaluating a heterozygous strain of the target population with separate applications of the first toxin and the second toxin; and

utilizing the second toxin to manage the pest population if the separate applications of the first toxin and the second toxin are at least as toxic to the heterozygous strain as to the susceptible strain.

39. A method in accordance with Claim 37 wherein the pest population comprises at least one of a weed population, a bacterial population, an insect population, a fungus population, and a virus population.

40. A method in accordance with Claim 37 wherein the pest population comprises a population of disease contributing organisms living in a body of a mammal.

41. A method for utilizing a pest management system to manage a pest population, the system including a plurality of molecules, said method comprising:

determining a susceptible strain in the pest population, the susceptible strain susceptible to a first toxin;

selecting for a resistant strain in the pest population, the resistant strain resistant to the first toxin;

evaluating the resistant strain to determine a second toxin that is more toxic to the resistant strain than to the susceptible strain;

evaluating a heterozygous strain of the target population with separate applications of the first toxin and the second toxin; and

utilizing the second toxin to manage the pest population if the separate applications of the first toxin and the second toxin are at least as toxic to the heterozygous strain as to the susceptible strain.

42. A method in accordance with Claim 41 wherein the pest population comprises at least one of a weed population, a bacterial population, an insect population, a fungus population, and a virus population.

43. A method in accordance with Claim 41 wherein the pest population comprises a population of disease contributing organisms living in a body of a mammal.

44. A method of utilizing a pest management system that includes a plurality of molecules, said method comprising:

selecting a pest population;

obtaining a first strain in the pest population, the first strain susceptible to a first toxin;

obtaining a second strain in the pest population, the second strain resistant to the first toxin;

evaluating the efficacy of the second strain with multiple molecules in the system to determine multiple second toxins that are more toxic to the second strain than to the first strain;

evaluating the efficacy of a third strain of the pest population with separate applications of the first toxin and at least one second toxins, the third strain heterozygous for resistance to the first toxin; and

selecting at least one of the at least one second toxin to manage the pest population based on the performance of the selected at least one of the at least one second toxin in the second strain evaluation and in the third strain evaluation.

45. A method in accordance with Claim 44 further comprising prioritizing the second toxins to determine an advancement order for advancement to additional evaluation regarding pest management.

46. A method in accordance with Claim 44 further comprising:

evaluating the efficacy of the third strain with the first toxin and the at least one second toxin applied at the same time; and

selecting at least one of the at least one second toxin to manage the pest population based on the performance of the selected at least one of the at least one second toxin if the application of the first toxin and the second toxin at the same time are at least as toxic to the third strain as to the first strain.

47. A method in accordance with Claim 44 wherein selecting a pest population comprises selecting at least one of a weed population, a bacterial population, an insect population, a fungus population, and a virus population.

48. A method in accordance with Claim 44 wherein selecting a pest population comprises selecting a population of disease contributing organisms living in a body of a mammal.

49. A method in accordance with Claim 44 wherein the toxins are delivered to the pest population utilizing at least one of sprays, pellets, powders, baited or non-baited traps, and transgenic organisms.

50. A method of utilizing a pest management system that includes a plurality of molecules, said method comprising:

selecting a pest population;

obtaining a first strain in the pest population, the first strain susceptible to a first toxin;

obtaining a second strain in the pest population, the second strain resistant to the first toxin;

evaluating the efficacy of the second strain with multiple molecules in the system to determine multiple second toxins that are more toxic to the second strain than to the first strain;

evaluating the efficacy of the third strain with the first toxin and at least one second toxin applied at the same time; and

selecting at least one of the at least one second toxin to manage the pest population based on the performance of the selected at least one of the at least one second toxin if the application of the first toxin and the second toxin at the same time are at least as toxic to the third strain as to the first strain.

51. A method in accordance with Claim 50 further comprising prioritizing the second toxins to determine an advancement order for advancement to additional evaluation regarding pest management based on their performance in the third strain efficacy evaluation.

52. A method in accordance with Claim 50 wherein selecting a pest population comprises selecting at least one of a weed population, a bacterial population, an insect population, a fungus population, and a virus population.

53. A method in accordance with Claim 50 wherein selecting a pest population comprises selecting a population of disease contributing organisms living in a body of a mammal.

54. A method in accordance with Claim 50 wherein the toxins are delivered to the pest population utilizing at least one of sprays, pellets, powders, baited or non-baited traps, and transgenic organisms.

55. A method of evaluating a molecule for negative cross resistance, said method comprising using a *Rst(2)DDT* locus in Drosophila melanogaster as a resistance locus for use in negative cross resistance screens for molecules capable of controlling metabolic resistance to insecticides.

56. A method in accordance with Claim 55 further comprising:

evaluating the efficacy of a resistant Drosophila melanogaster strain with a plurality of molecules to determine a toxin that is more toxic to the resistant strain than to a susceptible strain; and

evaluating the efficacy of a heterozygous Drosophila melanogaster strain with the first toxin and the second toxin applied at the same time.

57. A method in accordance with Claim 55 further comprising assigning a priority rating to the second toxin if the application of the first toxin and the second toxin at the same time are at least as toxic to the heterozygous strain as to the susceptible strain.

58. A method in accordance with Claim 55 further comprising:

evaluating the efficacy of a heterozygous Drosophila melanogaster strain with separate applications of the first toxin and the second toxin; and

assigning a priority rating to the second toxin if the separate applications of the first toxin and the second toxin are at least as toxic to the heterozygous strain as to the susceptible strain.

59. A method in accordance with Claim 55 further comprising evaluating the efficacy of a homozygous Drosophila strain with compounds to determine whether a potential negative cross resistance factor provides field-resistance to at least one of chlordane, lindane, DDT, and imidacloprid.

60. A method in accordance with Claim 55 further comprising evaluating the efficacy of a heterozygous Drosophila strain with compounds to determine whether a potential negative cross resistance factor provides field-resistance to at least one of chlordane, lindane, DDT, and imidacloprid.

61. A method for evaluating molecules for negative cross resistance, said method comprising using a putative target site locus in Drosophila melanogaster as a resistance locus for use in negative cross resistance screens for molecules capable of controlling metabolic resistance to insecticides.

62. A method in accordance with Claim 61 wherein the target site locus is for at least one of Rst(2)DDT, acetylcholinesterases, voltage-gated sodium channels, GABA receptors, Rdl or Resistance to Dieldrin in Drosophila, esterases,

cytochrome P450s, neurotransmitter uptake channels, cation and ion channel, aromatic biogenic amine receptors, Glutathione-S-Transferases, and CYP6G1.

63. A method in accordance with Claim 61 further comprising:

evaluating chemicals using the resistance locus;

identifying candidate chemicals as those that function as NCR factors; and

testing the candidate chemicals on a target population in a native setting.

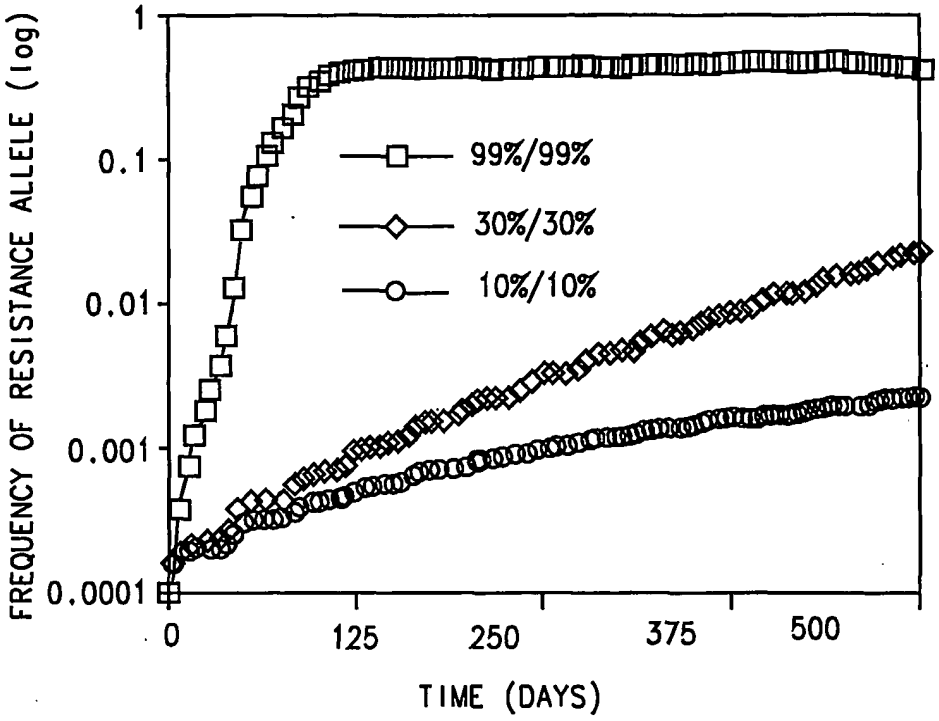

FREQUENCY OF RESISTANCE ALLELE (log)
1
0.1
0.01
0.001
0.0001
99%/99%
30%/30%
10%/10%
0
125
250
375
500
TIME (DAYS)

FIG. 1

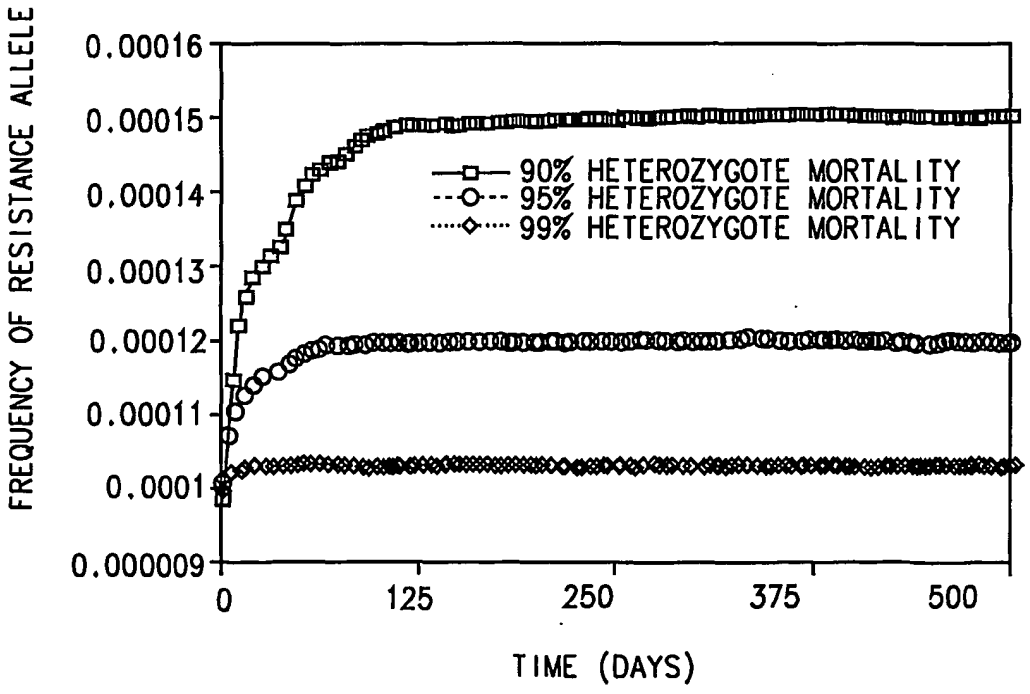

FREQUENCY OF RESISTANCE ALLELE
0.00016
0.00015
0.00014
0.00013
0.00012
0.00011
0.0001
0.000009
90% HETEROZYGOTE MORTALITY
95% HETEROZYGOTE MORTALITY
99% HETEROZYGOTE MORTALITY
0
125
250
375
500
TIME (DAYS)

FIG. 2

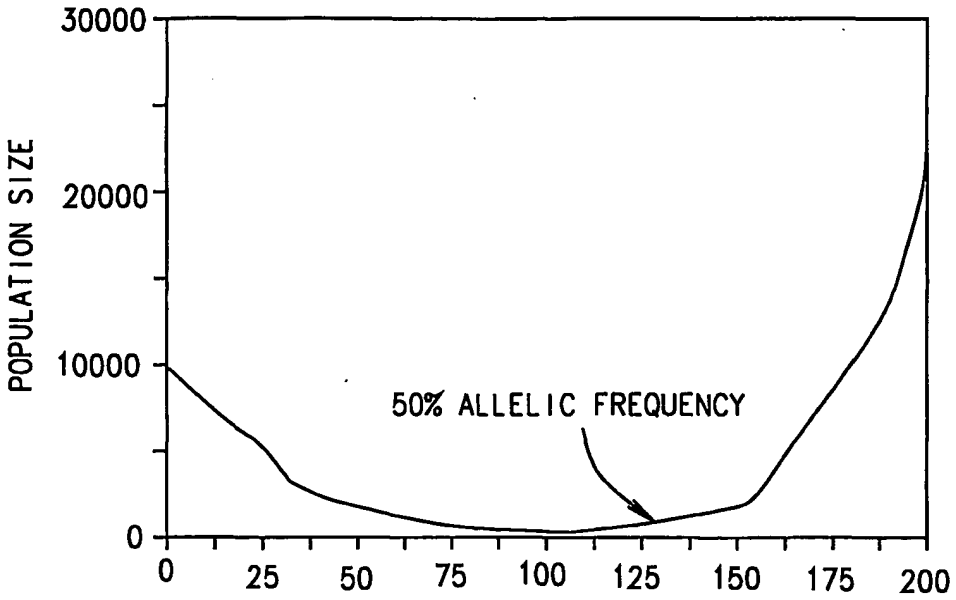

30000
20000
10000
0
POPULATION SIZE
50% ALLELIC FREQUENCY
0
25
50
75
100
125
150
175
200

FIG. 3

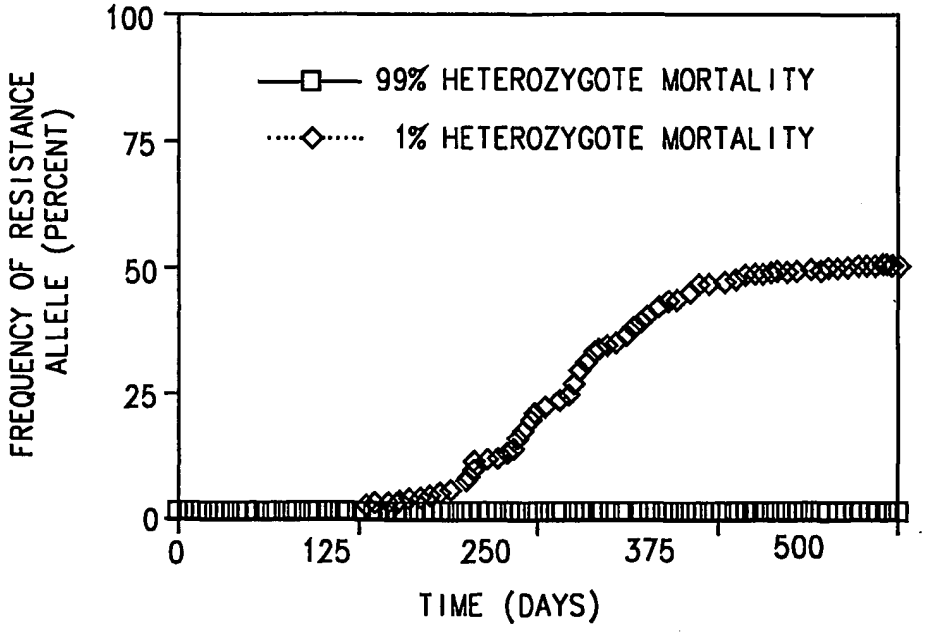

100
75
50
25
0
FREQUENCY OF RESISTANCE ALLELE (PERCENT)
99% HETEROZYGOTE MORTALITY
1% HETEROZYGOTE MORTALITY
0
125
250
375
500
TIME (DAYS)

FIG. 4

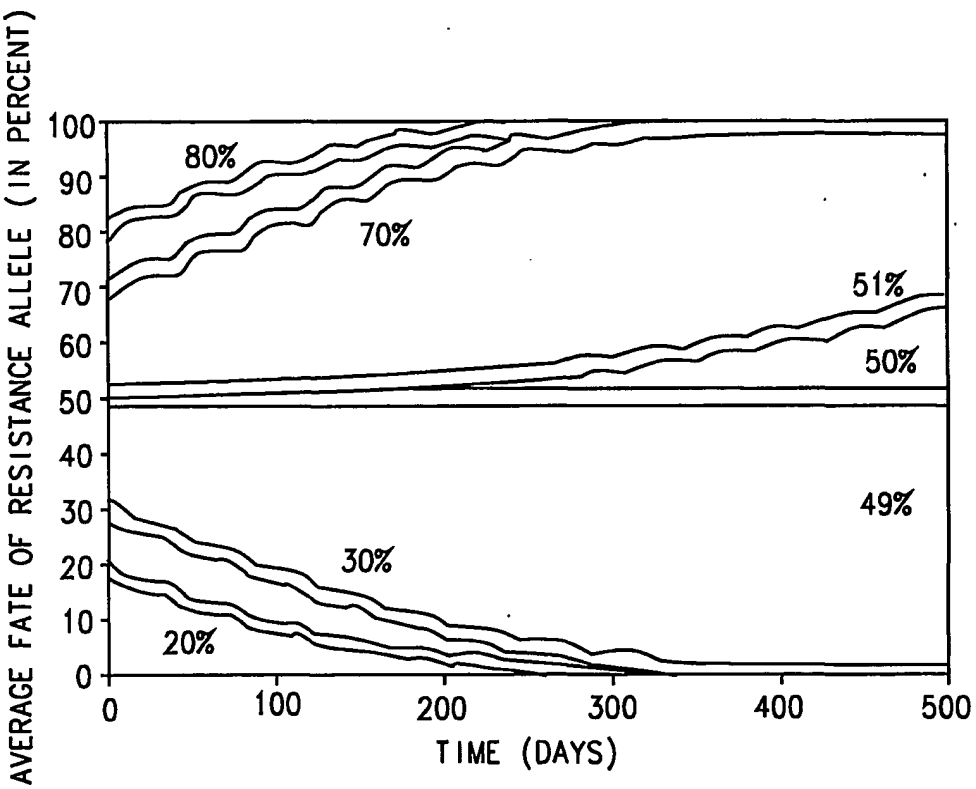

AVERAGE FATE OF RESISTANCE ALLELE (IN PERCENT)
100
90
80
70
60
50
40
30
20
10
0
80%
70%
51%
50%
49%
30%
20%
0
100
200
300
400
500
TIME (DAYS)

FIG. 5

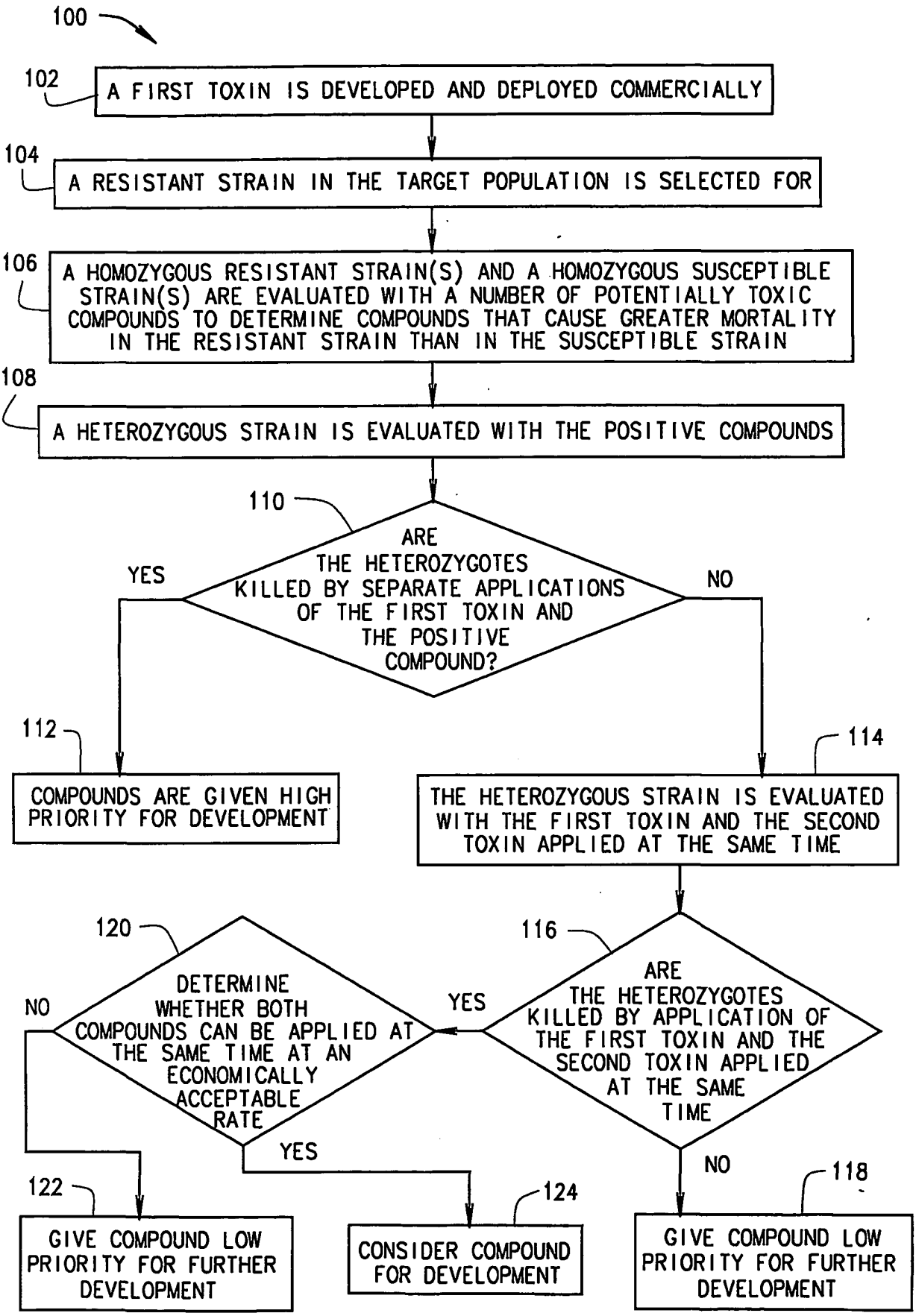

100
102
A FIRST TOXIN IS DEVELOPED AND DEPLOYED COMMERCIALLY
104
A RESISTANT STRAIN IN THE TARGET POPULATION IS SELECTED FOR
106
A HOMOZYGOUS RESISTANT STRAIN(S) AND A HOMOZYGOUS SUSCEPTIBLE STRAIN(S) ARE EVALUATED WITH A NUMBER OF POTENTIALLY TOXIC COMPOUNDS TO DETERMINE COMPOUNDS THAT CAUSE GREATER MORTALITY IN THE RESISTANT STRAIN THAN IN THE SUSCEPTIBLE STRAIN
108
A HETEROZYGOUS STRAIN IS EVALUATED WITH THE POSITIVE COMPOUNDS
110
ARE THE HETEROZYGOTES KILLED BY SEPARATE APPLICATIONS OF THE FIRST TOXIN AND THE POSITIVE COMPOUND?
YES
NO
112
COMPOUNDS ARE GIVEN HIGH PRIORITY FOR DEVELOPMENT
114
THE HETEROZYGOUS STRAIN IS EVALUATED WITH THE FIRST TOXIN AND THE SECOND TOXIN APPLIED AT THE SAME TIME
116
ARE THE HETEROZYGOTES KILLED BY APPLICATION OF THE FIRST TOXIN AND THE SECOND TOXIN APPLIED AT THE SAME TIME
YES
NO
118
GIVE COMPOUND LOW PRIORITY FOR FURTHER DEVELOPMENT
120
DETERMINE WHETHER BOTH COMPOUNDS CAN BE APPLIED AT THE SAME TIME AT AN ECONOMICALLY ACCEPTABLE RATE
NO
YES
122
GIVE COMPOUND LOW PRIORITY FOR FURTHER DEVELOPMENT
124
CONSIDER COMPOUND FOR DEVELOPMENT

FIG. 6

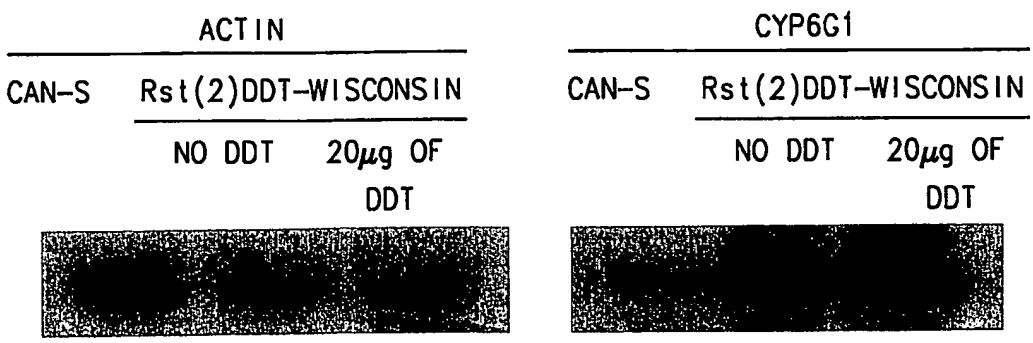

ACTIN
CAN-S
Rst(2)DDT-WISCONSIN
NO DDT
20μg OF DDT
CYP6G1
CAN-S
Rst(2)DDT-WISCONSIN
NO DDT
20μg OF DDT

FIG. 7

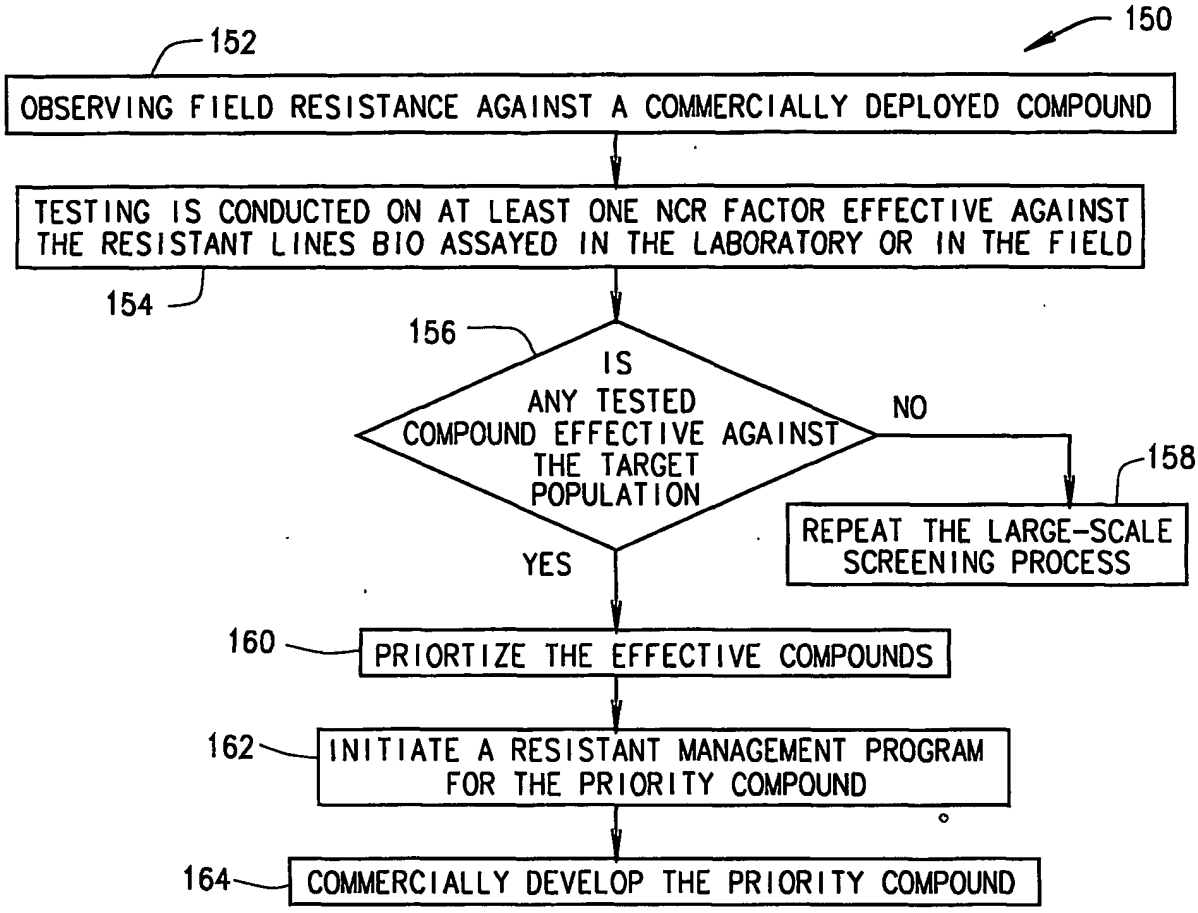

150
152
OBSERVING FIELD RESISTANCE AGAINST A COMMERCIALLY DEPLOYED COMPOUND
TESTING IS CONDUCTED ON AT LEAST ONE NCR FACTOR EFFECTIVE AGAINST THE RESISTANT LINES BIO ASSAYED IN THE LABORATORY OR IN THE FIELD
154
156
IS ANY TESTED COMPOUND EFFECTIVE AGAINST THE TARGET POPULATION
NO
158
REPEAT THE LARGE-SCALE SCREENING PROCESS
YES
160
PRIORTIZE THE EFFECTIVE COMPOUNDS
162
INITIATE A RESISTANT MANAGEMENT PROGRAM FOR THE PRIORITY COMPOUND
164
COMMERCIALLY DEVELOP THE PRIORITY COMPOUND

FIG. 8

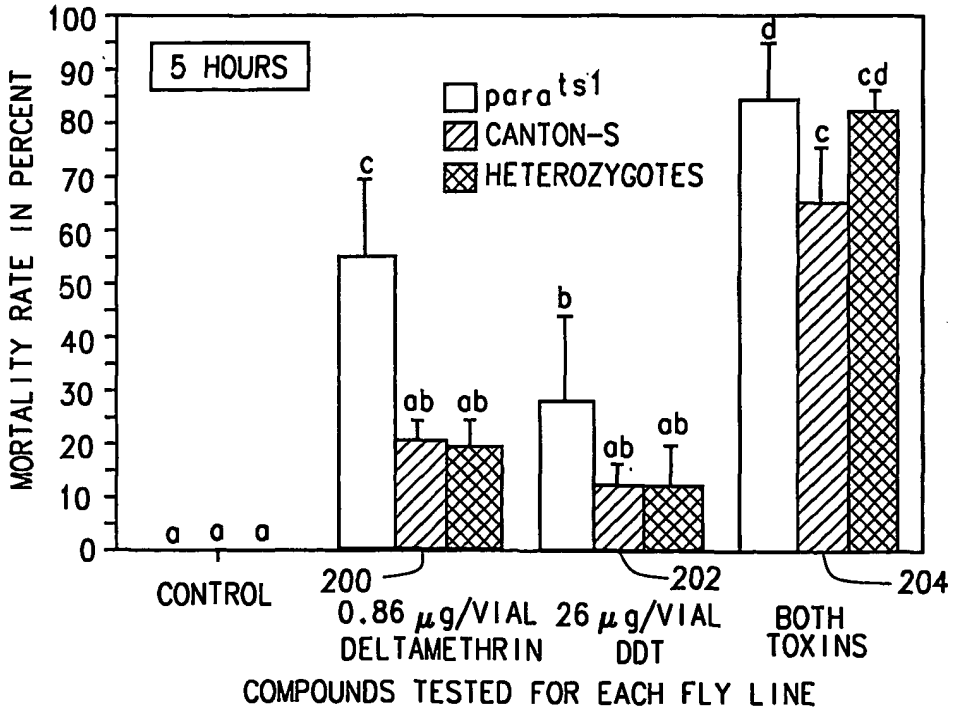

5 HOURS
para ts1
CANTON-S
HETEROZYGOTES
MORTALITY RATE IN PERCENT
100
90
80
70
60
50
40
30
20
10
0
a a a
c
ab ab
b
ab ab
d
c
cd
CONTROL
200
202
204
0.86 μg/VIAL DELTAMETHRIN
26 μg/VIAL DDT
BOTH TOXINS
COMPOUNDS TESTED FOR EACH FLY LINE

FIG. 9